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(54) SnRNP-A antigen and fragments thereof.

(57) The present invention relates to a protein antigen which is reactive towards an auto-antibody which is associated with an auto-immune disease, and the use of this protein in diagnostic tests relating to auto-immune diseases.

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snRNP-A antigen and fragments thereof.

The invention relates to a protein antigen which is reactive towards an auto-antibody which is associated with an auto-immun disease, and the use of this protein in diagnostic tests relating to auto-immune diseases.

In healthy humans and animals, after the intrusion of a foreign substance (the antigen) the body will attempt to attack this antigen by generating specific antibodies which are directed against this antigen. Most individuals are in general tolerant to substances which occur in their own body. Some individuals on th other hand generate antibodies against endogenous substances, tissues, or components. Such antibodies (auto-antibodies) cause great damage to the organs which contain these endogenous substances. The development of the associated auto-immune disease is in general very slow (a matter of years) and this hampers timely clinical diagnosis and treatment to a high degree. Diagnosis can generally only be mad after appreciable damage has already been caused to the body. Earlier research has shown that many of these auto-antibodies are syndrome-specific, i.e. the disease seems to be characterized by the occurrence of specific auto-antibodies.

Furthermore, it appears from recent research that these specific auto-antibodies can often be detected in the serum of a patient long before the clinical diagnosis can be made with certainty. The auto-antibodi s therefore predict, as it were, which disease is developing.

The timely detection of these auto-antibodies in the patient's serum is the more important because the patient's treatment can then be initiated earlier, thereby delaying, or even preventing, the often serious damage during the later phase of the disease.

Patients with an auto-immune disease possess auto-antibodies directed against one or more protein antigens, such as small protein molecules which occur in the cell nucleus and which are complexed with ribonucleic acid, such as the so-called snRNPS (small nuclear ribonucleoproteins).

A snRNP protein and fragments thereof having a well defined amino acid sequence have now been found, which render it possible to diagnose immune diseases, especially systemic lupus erythematosus (SLE) and mixed connective tissue disease (MCTD), at an early stage.

The invention therefore relates to a protein with the structure as shown in formula 1 which protein is substantially free from other natural material, and to fragments thereof which are immunochemically reactiv-, and to peptides which contain such fragments.

The invention furthermore relates to preparative methods for such a protein, fragment or peptide, to diagnostic methods for the detection of auto-immune antibodies with the aid of such a protein, fragment, or peptide and to test kits for carrying out such diagnostic methods, and to pharmaceutical compcsitions for combating auto-immune diseases, especially SLE and MCTD, which compositions contain such a protein, fragment or peptide.

The invention furthermore relates to DNA which codes for the protein, the fragments and the peptides according to the invention, to a vector which contains the DNA, and to a host which contains such a vector.

The protein according to the invention is known in the literature as the U_1-snRNP-A protein. This protein, however, has been insufficiently purified in the literature. Structure elucidation has been impossible hitherto.

By using a DNA clone which contains the nucleotide sequence coding for human U_1-snRNP-A protein, and which clone was obtained using recombinant DNA techniques known per se, it was possible to elucidate, by techniques known per se, the amino acid sequence of the U_1-snRNP-A protein, as is shown in formula 1.

Some fragments of the protein according to the invention react specifically with auto-antibodies against the U_1-snRNP-A protein. Surprisingly, it has now also been found that other fragments of the protein react with auto-antibodies against the U_2-snRNP-B" protein and the Sm protein, two other snRNP proteins. Th presence of antibodies against the Sm protein form an indication that the patient is developing SLE, whilst the occurrence of antibodies against the U_1-snRNP-A and U_2-snRNP-B" proteins provides an indication of MCTD.

The fragments of the protein of formula 1 which react with antibodies against U_1-snRNP-A, U_2-snRNP-B" and Sm protein have the aminoacid sequences shown in formula 2, 3, and 4 respectively. Th invention also relates to smaller fragments of th s amino acid sequences mentioned provided thes smaller fragments are still immunochemically reactive, such as the amino acid sequence of formula 2a (being a smaller fragment of th p ptide of formula 2) and the amino acid sequenc of formula 3a (being a smaller fragment of the peptid of formula 3). The invention further relates to peptides which contain such

fragments.

The preparation of these immunochemically reactive protein fragments according to the invention follows known routes, such as chemical synthesis or recombinant DNA techniques.

The protein, the fragments and the peptide according to the invention can be used for the detection of the auto-antibodies mentioned using diagnostic methods known per se for the determination of antibodies by means of antigens reactive with them.

Both homogeneous and heterogeneous diagnostic tests are suitable for this purpose. Use can thus be made of sandwich type tests or of an agglutination test in which, if desired, an inhibition or competition reaction is used.

Suitable tests are those in which a solid phase is used, such as the inner wall of a microassay well, a tube or capillary, a membrane, filter, test strip or the surface of a particle to which an antibody or antigen is bound. For the detection, use is thus made of an antigen or antibody which is provided with a label, such as a radio-active isotope, a dyestuff, metal sol such as a gold sol, an enzyme, or another known label. Methods for the preparation of labelled antigens or antibodies are generally known.

Moreover, the protein, fragments thereof, and peptides which contain such fragments, can be used in suitable pharmaceutical dosage forms against auto-immune diseases. For example, purified U_1snRNP-A antigen can be injected intravenously into a patient who is producing antibodies against this antigen. One should administer to a human preferably 1-1000 nanomoles of said A-antigen.

Administration of such a preparation via the circulatory system of a patient with an auto-immune disease results in an antigen-antibody complex formation which can decrease further attack on the abovementioned tissues. The diagnostic methods mentioned can be used to determine the precise quantity of auto-antibodies circulating in the blood. The quantity of the preparation to be administered is determined by the result of said test. The invention is illustrated by reference to the following Examples.

Example I

Nucleotide sequence and inferred amino acid sequence of the A protein

Starting from the material lodged with the Central Office for Mould Cultures in Baarn, The Netherlands under number CBS 617.87, the nucleotide sequence of the cDNA inserts has been determined using the so-called dideoxy method described by Sanger et al. (PNAS, 74, 5463-5467, 1977). This nucleotide sequence is shown in formula 5. The initiation codon ATG is located at nucleotide position 126.

The stop codon TAG is located at nucleotide position 972, followed by a non-coding region of 223 nucleotides. The inferred amino acid sequence of 282 amino acids for the A protein (formula 1) gives a molecular weight of 31.2 kd, which is in good agreement with the observed molecular weight of 32 kd for the A protein analyzed using a SDS-polyacrylamide gel.

Example II

Mapping of an antigenic determinant of the A protein

The amino acid sequence of the A protein inferred from the nucleotide sequence as given, has an interesting distribution of charged and aromatic amino acids. These can be assigned to two regions, separated by a segment with a very high proline content (amino acid 140 to 206 in figure 1). Most of the charged and aromatic amino acids are located on the N-terminal part of the protein. The amino acid sequence of the A protein, according to Formula 1, can be split into two parts, namely amino acid 1 to 171 and amino acid 172 to 282. The DNA sequences coding for these sequences, are each placed separately in a suitable expression vector with recombinant DNA techniques know per se. The proteins synthesized by this expression vector are then brought into contact with patient serum containing auto-antibodies via an immunoblotting experiment. Patient serum containing anti-Sm-antibodies reacts specifically with the N-terminal part (amino acid 1 to 171) of the A protein. In this N-terminal part there is a cluster of eleven charged amino acids which represent a hydrophilic section of the A protein (amino acid 103 to 112 in

formula 1). Hydrophilicity analysis using the Hopp and Woods method supports this site determination. This sequence Glu - Arg - Asp - Arg - Lys - Arg - Glu - Lys - Arg - Lys in the A protein thus forms an epitope which is recognized by patient serum which contains anti-Sm antibodies. Moreover, another unexpected aspect relating to the amino acid sequence of the A protein emerges from this immunoblotting experiment. Both patient serum containing anti-B''-antibodies and patient serum containing anti-A-antibodies react in an immunoblotting experiment with the C-terminal part of the A protein (amino acid 172 to 282 in formula 1). If the inferred amino acid sequence of the A protein is laid in a line with the known amino acid sequence of B'' protein, on the grounds of this overlap it can then be determined by exclusion that the specific epitope responsible for the antigen character with respect to anti-A-antibodies is located in the A protein in the region of amino acid 172 to 207 according to formula 1; this sequence represents a strongly hydrophilic area, and hydrophilicity analysis using the Hopp and Woods method supports this epitope mapping. This also establishes that the epitope which cross-reacts with anti-B''-antibody is located in the region of amino acid 207 to 282 in formula 1.

Example III

Enzyme Immuno Assay (EIA)

An EIA was set up in which was tested 480 sera from patients with connective tissue diseases. Simultaneously, the same serum samples were tested on immunoblots with eukaryotic nuclear proteins as antigen. The intensity of the reaction on these blots was scored on an arbitrary scale ranging from "very weak" to "strong". When sera were thus grouped on basis of increasing titer of antibodies against eukaryotic A or B'' proteins, the signals obtained in the EIA appeared to correlate perfectly with the blotting results; for the two antigens tested, an increased blotting signal corresponded with an increased EIA reading. When patients were subsequently grouped on basis of their diagnosis, it appeared that this simpl and sensitive EIA allowed to distinguish between patients with definite SLE and patients with MCTD. Especially the presence of antibodies against the A protein appeared to be diagnostic for MCTD, since they do not, or in very low titers, occur in definite SLE patients.

Claims

1. A protein with the structure as shown in formula 1, which protein is substantially free from other natural material, and fragments thereof which are immunochemically reactive and peptides which contain such fragments.

2. Amino acid sequences according to claim 1 of the formula 2 and immunochemically reactive fragments thereof and peptides which contain such sequences or fragments thereof.

3. Amino acid sequences according to claim 1 of the formula 3 and immunochemically reactive fragments thereof and peptides which contain such sequences or fragments thereof.

4. Amino acid sequences according to claim 1 of the formula 4 and immunochemically reactive fragments thereof and peptides which contain such sequences or fragments thereof.

5. Amino acid sequence according to claim 1 or 2 of the formula 2a.

6. Amino acid sequence according to claim 1 or 3 of the formula 3a.

7. DNA coding for the fragments, the peptides, and the protein according to claim 1.

8. Vector which contains DNA according to claim 7.

9. Host which contains the vector according to Claim 8.

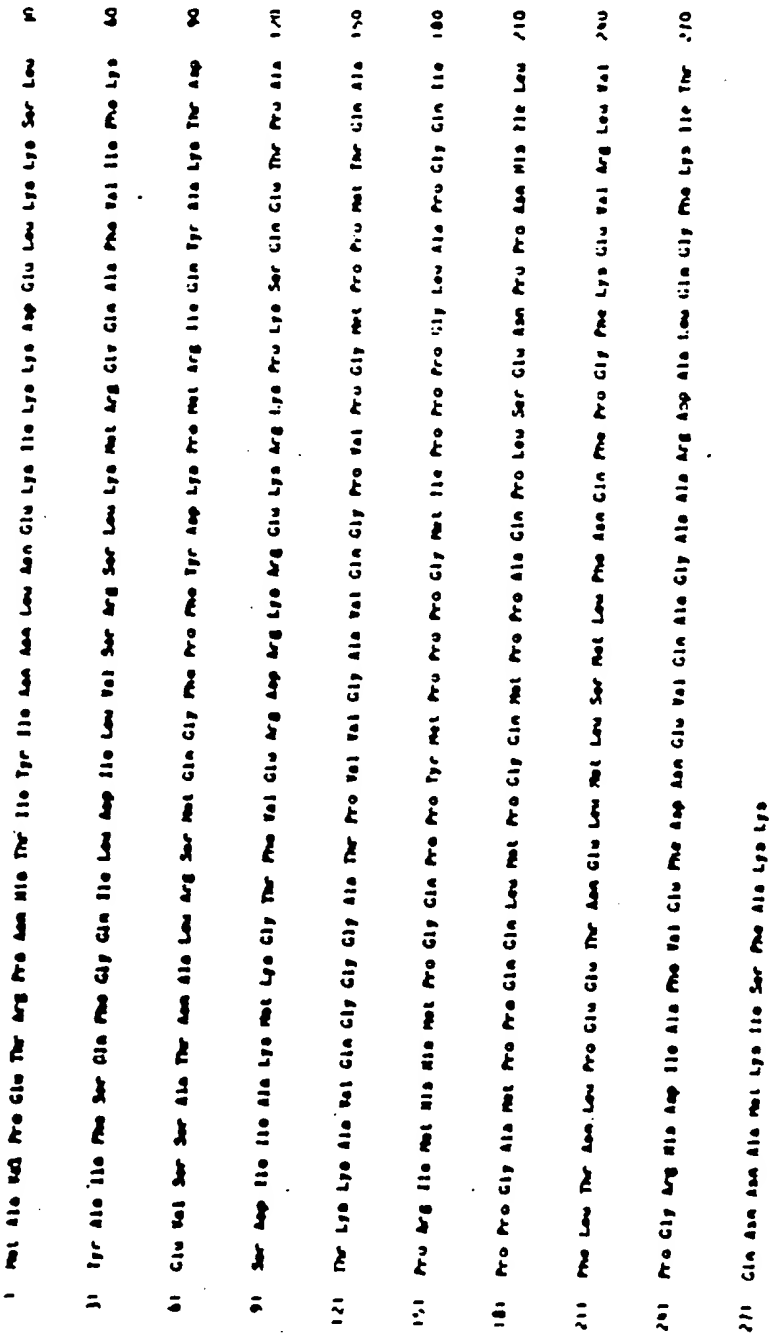
10. Method for the preparation of the protein according to Claim 1, and of fragments thereof which are immunochemically reactive and of peptides which contain such fragments, characterized in that the protein, the fragments, and the peptides are prepared according to a method known per se using peptide synthesis techniques and/or recombinant DNA techniques.

11. Method for the determination of an auto-antibody in a sample, characterized in that, use is made of the protein according to Claim 1 or a fragment thereof which is immunochemically reactive or a peptide which contains such a fragment in which at least one part of the protein, fragment, or peptide is provided with a label.

12. Test kit for the determination of an auto-antibody with the characteristic that the test kit, along with the usual reagents for these determinations, also contains a protein according to Claim I or a fragment thereof which is immunochemically reactive or a peptide which contains such a fragment in which at least on part of the protein fragment or peptide is provided with a label.

13. Pharmaceutical preparation containing, along with the usual additions, the protein according to Claim 1 as active component, or a fragment thereof which is immunochemically reactive, or a peptide which contains such a fragment.

Formula 1



Formula 2

Pro Pro Gly Leu Ala Pro Gly Gln Ile Pro Pro Gly Ala Met Pro Pro Gln Gln Leu Met Pro Gly Gln Met Pro Pro Ala Gln Pro Leu Ser Gln Asn Pro Pro Asn

Formula 2a

Pro Pro Gly Leu Ala Pro Gly Gln Ile Pro Pro Gly Ala Met

Formula 3

Asn His Ile Leu

Phe Leu Thr Asn Leu Pro Glu Glu Thr Asn Glu Leu Met Leu Ser Met Leu Phe Asn Gln Phe Pro Gly Phe Lys Glu Val Arg Leu Val

Pro Gly Arg His Asp Ile Ala Phe Val Glu Phe Asp Asn Glu Val Gln Ala Gly Ala Ala Arg Asp Ala Leu Gln Gly Phe Lys Ile Thr

Gln Asn Asn Ala Met Lys Ile Ser Phe Ala Lys Lys

Formula 3a

Pro Gly Phe Lys Glu Val Arg Leu Val Pro Gly Arg His Asp Ile Ala Phe Val Glu Phe Asp Asn Glu Val Gln Ala Gly Ala Ala Arg Asp Ala Leu Gln

Formula 4

Glu Arg Asp Arg Lys Arg Glu Lys Arg Lys

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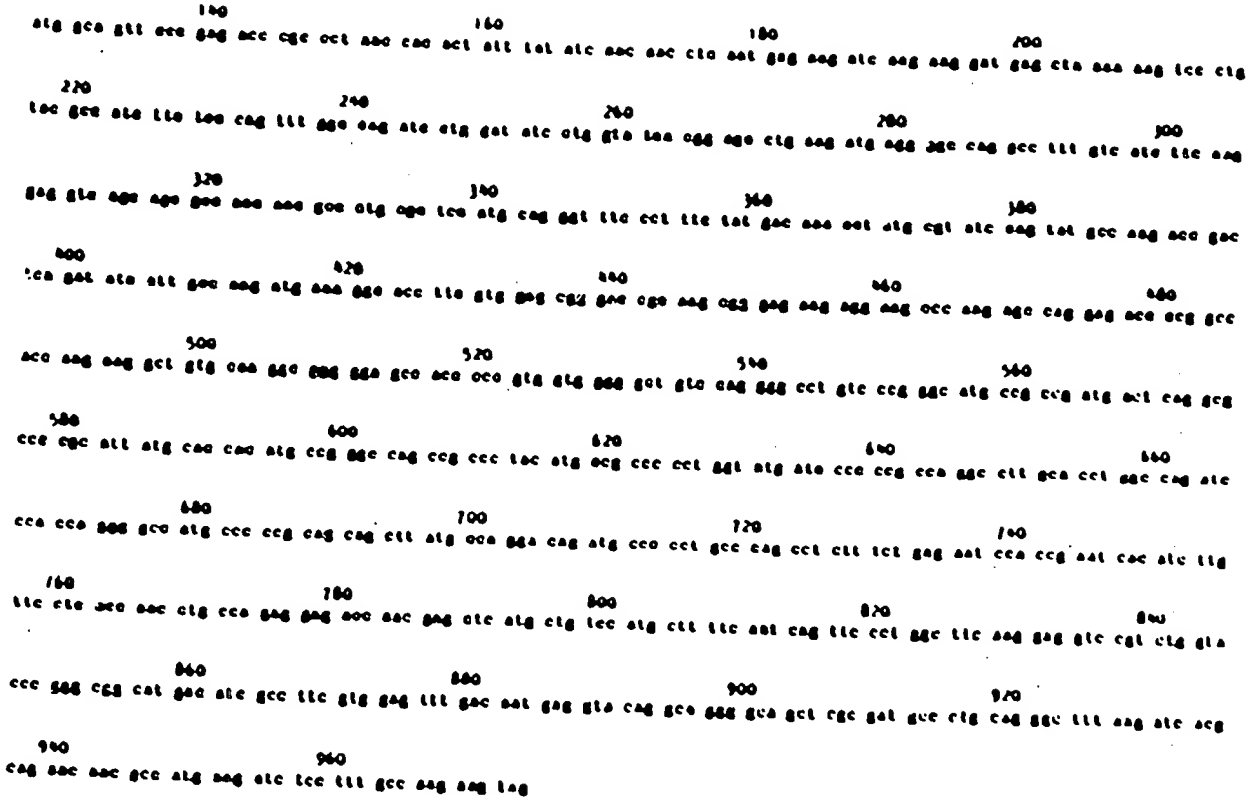
Formula 5



Formula 1

1	Met Ala Val Pro Glu Thr Arg Pro Asn His Thr Ile Tyr Ile Asn Asn Leu Asn Glu Lys Ile Lys Lys Asp Glu Leu Lys Lys Ser Leu	30
31	Tyr Ala Ile Phe Ser Gln Phe Gly Gln Ile Leu Asp Ile Leu Val Ser Arg Ser Leu Lys Met Arg Gly Gln Ala Phe Val Ile Phe Lys	60
61	Glu Val Ser Ser Ala Thr Asn Ala Leu Arg Ser Met Gln Gly Phe Pro Phe Tyr Asp Lys Pro Met Arg Ile Gln Tyr Ala Lys Thr Asp	90
91	Ser Asp Ile Ile Ala Lys Met Lys Gly Thr Phe Val Glu Arg Asp Arg Lys Arg Glu Lys Arg Lys Pro Lys Ser Gln Glu Thr Pro Ala	120
121	Thr Lys Lys Ala Val Gln Gly Gly Gly Ala Thr Pro Val Val Gly Ala Val Gln Gly Pro Val Pro Gly Met Pro Pro Met Thr Gln Ala	150
151	Pro Arg Ile Met His His Met Pro Gly Gln Pro Pro Tyr Met Pro Pro Pro Gly Met Ile Pro Pro Pro Gly Leu Ala Pro Gly Gln Ile	180
181	Pro Pro Gly Ala Met Pro Pro Gln Gln Leu Met Pro Gly Gln Met Pro Pro Ala Gln Pro Leu Ser Glu Asn Pro Pro Asn His Ile Leu	210
211	Phe Leu Thr Asn Leu Pro Glu Glu Thr Asn Glu Leu Met Leu Ser Met Leu Phe Asn Gln Phe Pro Gly Phe Lys Glu Val Arg Leu Val	240
241	Pro Gly Arg His Asp Ile Ala Phe Val Glu Phe Asp Asn Glu Val Gln Ala Gly Ala Ala Arg Asp Ala Leu Gln Gly Phe Lys Ile Thr	270
271	Gln Asn Asn Ala Met Lys Ile Ser Phe Ala Lys Lys	

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Formula 2

Pro Pro Gly Leu [illegible] Pro Gly Gln Ile Pro Pro Gly Ala Met Pro Pro Gln Gln Leu Met Pro Gly Gln Met Pro Pro Ala Gln Pro Leu Ser Glu Asn Pro Pro Asn

Formula 2a

Pro Pro Gly Leu Ala Pro Gly Gln Ile Pro Pro Gly Ala Met

Formula 3

Asn His Ile Leu

Phe Leu Thr Asn Leu Pro Glu Glu Thr Asn Glu Leu Met Leu Ser Met Leu Phe Asn Gln Phe Pro Gly Phe Lys Glu Val Arg Leu Val

Pro Gly Arg His Asp Ile Ala Phe Val Glu Phe Asp Asn Glu Val Gln Ala Gly Ala Ala Arg Asp Ala Leu Gln Gly Phe Lys Ile Thr

Gln Asn Asn Ala Met Lys Ile Ser Phe Ala Lys Lys

Formula 3a

Pro Gly Phe Lys Glu Val Arg Leu Val Pro Gly Arg His Asp Ile Ala Phe Val Glu Phe Asp Asn Glu Val Gln Ala Gly Ala Ala Arg Asp Ala Leu Gln

Formula 4

Glu Arg Asp Arg Lys Arg Glu Lys Arg Lys

Formula 5

140 160 180 200
atg gca gtt ccc gag acc cgc cct aac cac act att tat atc aac aac ctc aat gag aag atc aag aag gat gag cta aaa aag tcc ctg

220 240 260 280 300
tac gcc atc ttc tcc cag ttt ggc cag atc ctg gat atc ctg gta tca cgg agc ctg aag atg agg ggc cag gcc ttt gtc atc ttc aag

320 340 360 380
gag gtc agc agc gcc acc aac gcc ctg cgc tcc atg cag ggt ttc cct ttc tat gac aaa cct atg cgt atc cag tat gcc aag acc gac

400 420 440 460 480
tca gat atc att gcc aag atg aaa ggc acc ttc gtg gag cgg gac cgc aag cgg gag aag agg aag ccc aag agc cag gag acc ccg gcc

500 520 540 560
acc aag aag gct gtg caa ggc ggg gga gcc acc ccc gta gtg ggg gct gtc cag ggg cct gtc ccg ggc atg ccg ccg atg act cag gcg

580 600 620 640 660
ccc cgc att atg cac cac atg ccg ggc cag ccg ccc tac atg ccg ccc cct ggt atg atc ccc ccg cca ggc ctt gca cct ggc cag atc

680 700 720 740
cca cca ggg gcc atg ccc ccg cag cag ctt atg cca gga cag atg ccc cct gcc cag cct ctt tct gag aat cca ccg aat cac atc ttg

760 780 800 820 840
ttc ctc acc aac ctg cca gag gag acc aac gag ctc atg ctg tcc atg ctt ttc aat cag ttc cct ggc ttc aag gag gtc cgt ctg gta

860 880 900 920
ccc ggg cgg cat gac atc gcc ttc gtg gag ttt gac aat gag gta cag gca ggg gca gct cgc gat gcc ctg cag ggc ttt aag atc acg

940 960
cag aac aac gca atg aag atc tcc ttt gcc aag aag tag

European Patent Office

EUROPEAN SEARCH REPORT

Application Number

EP 88 20 2301

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, vol. 83, November 1986, pages 8689-8693, The National Academy of Sciences, Washington, D.C., US; R. REUTER et al.: "Immunization of mice with purified U1 small nuclear ribonucleoprotein (RNP) induces a pattern of antibody specificities characteristic of the anti-Sm and anti-RNP autoimmune response of patients with lupus erythematosus, as measured by monoclonal antibodies" * Whole article * ---	1-13	C 07 K 13/00 C 12 N 15/00 G 01 N 33/68 A 61 K 37/02 G 01 N 33/564
A	THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 259, no. 9, 10th May 1984, pages 5907-5914, The American Society of Biological Chemists, Inc., US; I. PETTERSSON et al.: "The structure of mammalian small nuclear ribonucleoproteins" * Whole article * ---	1-13	
A	THE EMBO JOURNAL, vol. 5, no. 13, 1986, pages 3509-3516, IRL Press Ltd, Oxford, GB; P. BRINGMANN et al.: "Purification of the individual snRNPs U1, U2, U5 and U4/U6 from hela cells and characterization of their protein constituents" * Pages 3509-3516; page 3510, figure 2; page 3513, figure 6 * --- -/-	1-13	TECHNICAL FIELDS SEARCHED (Int. Cl.4) C 07 K C 12 N G 01 N A 61 K C 12 P

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	27-01-1989	HERMANN R.R.W.

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
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T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
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A	THE EMBO JOURNAL, vol. 2, no. 7, 1983, pages 1129-1135, IRL Press Ltd, Oxford, GB; P. BRINGMANN et al.: "Purification of snRNPs U1, U2, U4, U5 and U6 with 2,2,7-trimethylguanosine-specific antibody and definition of their constituent proteins reacting with anti-Sm and anti-(U1)RNP antisera" * Whole article *		

TECHNICAL FIELDS SEARCHED (Int. Cl.4)

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